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A Study on genetic diversity and evolutionary analysis of *ECA1* Protein in different plant families

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Abstract

Calcium and manganese are two of the most important nutrients required by plants. Calcium/manganese uptake and accumulation is carried out by a large group of transporters. Among these, the P-type ATPases are considered most important. These ATPases have been divided into two categories i.e., P_{2A} and P_{2B} based on the absence and presence of the N terminal autoinhibitory domain respectively. *ECA1* is an important P_{2A}-type ATPase that is localized in the endosomal system and has crucial roles in calcium and manganese translocation. In this study, the phylogeny of *ECA1* protein within different plant families as well as conserved motifs putatively involved in Ca^{2+} ions binding was investigated. Phylogenetic analysis and diversity in predicted tertiary structures indicated that *ECA1* protein is functionally conserved but structurally diverged in different plant families. Moreover, some amino acids are found to be involved in Ca^{2+} ions binding in *ECA1* proteins of different plant species which gives the idea that the evolution of *ECA1* gene is monophyletic hence, indicating divergent evolution.

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Introduction

Plants require different metal ions to complete their life cycle. Generally, the number of macronutrients required by a plant is 100 to 300 fold higher than that of micronutrients such as Mn^{2+}, $Zn^{2+,}$ etc. Though required in very fewer amounts, micronutrients are equally essential for the growth and development of the plant. Growth is badly inhibited when the amount of macronutrients/micronutrients falls below or above the average value required by the plant. One of the important macronutrients is calcium which is required for a variety of cellular functions in living organisms. For instance, it acts as a secondary messenger and plays role in the signal transduction pathway. This pathway helps the plant cells to react against different biotic and abiotic stresses. Therefore, adequate levels of calcium in plants aid in the plant's ability to isolate infection hence referred as "plants first-line defense". Calcium is also mandatory for proper growth and development of a living body and is also essential for maintaining the structural rigidity of the plant cell wall and for controlling membrane structure and function (Wyn Jones and Lunt, 1967; Burstrom, 1968). Another key role of calcium in plants is to mitigate the heat stress effect by improving stomatal functions and other cellular processes. Insufficient calcium supply to the plants has effects such as poor root development, leaf necrosis, blossom end rot and water soaking (White and Broadley, 2003). The deterioration of cell membranes (results in loss of cell compounds which eventually leads to cell death) occurs due to insufficient calcium levels in plants. On the other hand, excessive calcium ions have certain damaging effects on the plants such as the development of tiny yellowish flecks or 'gold spots" in the cell walls around the calyx and shoulder of the fruits which are calcium oxalate crystals (De kreij *et al.*, 1992). Excess calcium in the rhizosphere may prevent germination of seeds, reduce plant growth rates and also cause the production of certain toxic compounds within cells which ultimately leads to nucleic acid damage (Case *et al.*, 2007). Plant cells, therefore, require a homeostatic concentration of calcium ions to function in intracellular signaling and to prevent injuries to the cells. Similarly, manganese (a micronutrient) is a part of the oxygen-evolving complex in photosystem II and is required for photosynthesis. This important cation is also involved in certain reactions such as oxidation-reduction, decarboxylation, and biosynthesis (Marschner, 1995). It is very important for the plant cellular machinery to tightly regulate the amount of calcium and manganese ions entering the cell. It is because at higher concentrations both ions are potentially toxic for the cell.

The tight regulation of calcium and manganese is achieved in the cells by highly advanced machinery, the buildup of proteins, which export Ca^{2+} and Mn^{2+} ions efficiently into organelles or out of the cell (Dodd *et al.*, 2006). These transporters can be divided into three major classes i.e., exchangers, channels, and ATPases (pumps). Exchangers are responsible for Calcium sequestration; however, they have a lower affinity for calcium ions as compared to calcium ATPases (pumps) (Kudla *et al.*, 2010). Calcium exchangers are secondary active transporters, which use energy from the flow of one ion down its concentration gradient to transport another ion against its concentration gradient (Monteith, 2007). "Channels" are proteins in the membrane which translocate calcium ions across the concentration gradient (Nagat *et al.*, 2004). Certain "channels" such as cyclic nucleotide-gated channels glutamate receptors have been identified in plants having roles in calcium translocations (Kudla *et al.*, 2010). The ATPases are specialized membrane-bounded proteins that play role in ionic homeostasis within the eukaryotic cell by actively pumping calcium/manganese ions in and out of the cell as well as to certain organelles (Shabala *et al.*, 2011). Broadly, ATPases are divided into classes such as P-class, V-class, F-class, and ABC superfamily. The P-type ATPases are further divided into P_{1A}, P_{3A}, $P_{5,}$ P_4 and P_{2-} types based on ionic specificities. For example, the P_2-type ATPases are specialized proteins responsible for the translocation of calcium ions across membranes. They use energy which is derived from the hydrolysis of Adenosine triphosphate (ATP) to transport calcium ions against the concentration

gradient. The P_2- type ATPases are broadly divided into two major families: Type P_{2A}-ATPases which lack an N terminal regulatory domain and type P_{2B}-ATPases which are characterized by the presence of N terminal autoinhibitory domain-containing Ca^{2+}/Cam binding site and phosphorylation site (Tuteja and Mahajan, 2007). There are four types- IIA Calcium ATPases (*ECA1*, *ECA2*, *ECA3,* and *ECA4*) identified in *Arabidopsis thaliana*, while three (*ECA1*, *ECA2,* and *ECA3*) have identified in *Oryza sativa*. The first P_{2A} - type ATPase studied was At*ECA1* (Liang *et al*., 1997), which was believed to have roles in the transport of calcium and manganese (Liang *et al*., 1997; Liang and Sze, 1998). This gene is composed of eight exons and seven introns (Fig. 1). Another important P_{2A}-type calcium ATPase is known as *ECA3* has been found to have important roles in calcium and manganese homeostasis in Arabidopsis (Mills *et al*., 2008). Despite all the advancements, still, a lot is to be explored about the molecular mechanisms involved in transporting these ions from the soil to the cytosol and from the cytosol to intracellular compartments such as Golgi bodies and Endoplasmic reticulum. The availability of many sequenced genomes greatly facilitate the investigation of the evolutionary history and diversity of many environmentally relevant gene families, such as the P type-II ATPases using phylogenetic methods. Currently, we do not have sufficient information concerning the evolutionary relationships of these proteins within different plant species. In this paper, twenty sequences of *ECA1* gene representatives from different species were analyzed using Maximum Likelihood and Bayesian evolutionary methods to construct the evolutionary relationship between different plant species. Furthermore, conserved motifs in *ECA1* protein were also analyzed. Also, the amino acids putatively involved in calcium-binding in *ECA1* proteins were analyzed through alignment programs.

Material and methods

Taxon sampling

A total of twenty sequences of *ECA1* gene from different plant species were retrieved from Genbank (http://www.ncbi.nlm.nih.gov/). All sequences were renamed as follows: Gene name: Accession number: Species name. The criteria for selection of the sequences were (a) all sequences should be full length (b) isolation or publication date should be established in the literature. All sequences with incomplete information were not designated for analysis.

Alignment and Sequence Processing

Sequences retrieved were aligned by multiple sequence alignment using Geneious version 8.1.9 (http://www.geneious.com). The sequences chosen were believed to span the confirmed *ECA1* gene across the plant kingdom. Those sequences which had protein level and transcript level evidence for the sequence over those inferred only from homology were given preference. To retrieve the sequence "BLAST" searches were conducted using annotated *Arabidopsis thaliana ECA1* sequence as a query. The searches were also conducted using the keywords "*ECA1* ATPase" and "P_{2A} type ATPases". After the alignment, sequences were manually inspected and cropped. To perform a phylogenetic analysis neighbour-joining method was used. The neighbour-Joining tree was drawn by using KHY algorithm, as it was devised best-fit model by using the J model test version 2.1.3 according to Akaike information criterion for the data. The final number of sequences in the filtered set was 20 sequences (Table 1) which were realigned. The final alignment was used for subsequent phylogenetic and protein analysis.

Model Selection

J-Model Test v. 2.1.7 (Posada, 2008) was used to select the simplest evolutionary model that competently fitted the sequence data. J-Model Test allows optimization of base trees for every individual model. We choose the best-fitting models according to Akaike Information Criterion (AIC) (Akaike, 1974), Bayesian Information Criterion (BIC) (Schwarz, 1978) and a Decision-Theoretic Performance-Based approach (DT).

Phylogenetic Analysis

For phylogenetic tree construction, twenty-one sequences (twenty representative sequences, one

outgroup) were used to conduct the Neighbor-joining analysis plugin in Geneious version 8.1.9. J. PhyML a Geneious plugin was used to infer the maximum likelihood phylogenetic trees (Geneious v. 8.1.6) (Kearse *et al.*, 2012). The best suitable method selected after applying the J-model test was the HKY + I + G. The bootstrap value set for this analysis is 1,000. In this analysis the settings are specified as, the proportion of invariable site (+I) is estimated, the Gamma distribution parameter is also estimated, and the number of substitution rate categories is set at 4. Nearest Neighbor Interchange (NNI) was used in the tree topology search operation (Guindon *et al.*, 2010).

Protein modelling

To conduct comparative protein structure modelling MODELLER was used. The protein sequence for model construction was uploaded on ModWeb (https://modbase.compbio.ucsf.edu/modweb/). The predicted 3D structure was viewed in Geneious v. 8.1.6.

Results

Phylogenetic analysis

Phylogenetic analysis of twenty *ECA1* isolates from different species (Table 1) using the neighbour-joining method resulted in four main clades (Fig. 2).

It can be inferred from the tree that plant species studied here are monophyletic rather than being polyphyletic on basis of *ECA1* gene. Interestingly, the species appeared together in a single clade that ultimately belongs to the same family. A close look at the tree indicates that it is divided into two main clades. Clade I is composed of monocots whereas, clade II is composed of dicots.

Table 1. Accession numbers of the *ECA1* sequences used.

Accession #	Taxa	Reference
XP_002889666.1	*Arabidopsis lyrata*	Drive *et al.*, 2009
XP_006417763.1	*Eutrema salsugineum*	Schmutz *et al.*, 2013
XP_009118418.1	*Brassica rapa*	Brassica rapa Annotation Release 101
XP_010557282.1	*Tarenaya hassleriana*	Tarenaya hassleriana Annotation Release 101
XP_011005011.1	*Populus euphratica*	Populus euphratica Annotation Release 100
XP_002314209.1	*Populus trichocarpa*	Shu *et al.*, 2012
XP_011089397.1	*Sesamum indicum*	Sesamum indicum Annotation Release 101
XP_004251293.1	*Solanum lycopersicum*	Solanum lycopersicum Annotation Release 102
XP_006363343.1	*Solanum tuberosum*	Solanum tuberosum Annotation Release 101
XP_009590446.1	*Nicotiana tomentosiformis*	Nicotiana tomentosiformis Annotation Release 101
XP_009777607.1	*Nicotiana sylvestris*	Nicotiana sylvestris Annotation Release 100
XP_003554341.1	*Glycine max*	Glycine max Annotation Release 102
XP_007162693.1	*Phaseolus vulgaris*	Schmutz *et al.*, 2013
XP_004493912.1	*Cicer arietinum*	Cicer arietinum Annotation Release 101
XP_004302810.1	*Fragaria vesca*	Fragaria vesca Annotation Release 101
XP_009340897.1	*Pyrus x bretschneideri*	Pyrus x bretschneideri Annotation Release 101
XP_008369823.1	*Malus domestica*	Malus domestica Annotation Release 101
XP_010920750.1	*Elaeis guineensis*	Elaeis guineensis Annotation Release 101
XP_008810508.1	*Phoenix dactylifera*	Phoenix dactylifera Annotation Release 101
XP_010228776.1	*Brachypodium distachyon*	Brachypodium distachyon Annotation Release 102

A close relationship exists between both the clades as indicated by short branch lengths. Clade I is composed of three species namely *Barchypodium distachyon*, *Phoenix dactylifera*, and *Elaeis guineensis* which are all monocotyledons. Within this clade, *B. distachyon*, branched out separately whereas, *P. dactylifera*, and *E. guineensis* are grouped on basis of different plant families. Clade II is composed of species from seven different families (each indicated in different colours) and is divided into three subclades. The first clade is composed of families Rosaceae and Fabacaeae. Whereas, the second clade is composed of families Salicaceae, Cleomaceae and Brassicaeae. The last clade is composed of families Solanaceae and Pedialaceae. This diversification of families indicated the close

genetic relationships between them and is found to be consistent with the previous taxonomic analysis.

Conserved motifs

The sequence of amino acids that are believed to be conserved in all P_2-type ATPases in earlier studies were also analyzed. It indicated that events of evolutions and mutations did not alter the sequence of important motifs that are required for the functioning of this protein. The presence of the same motifs further suggests that the evolution of *ECA1* gene is monophyletic. Overall, it can be concluded that changes that occurred at the nucleotide level did not result in any significant effect on the tertiary structures of *ECA1* protein. As a result, the structure remained unchanged. Furthermore, conserved and variable regions of the *ECA1* gene provide its ancestral relationships and divergence respectively.

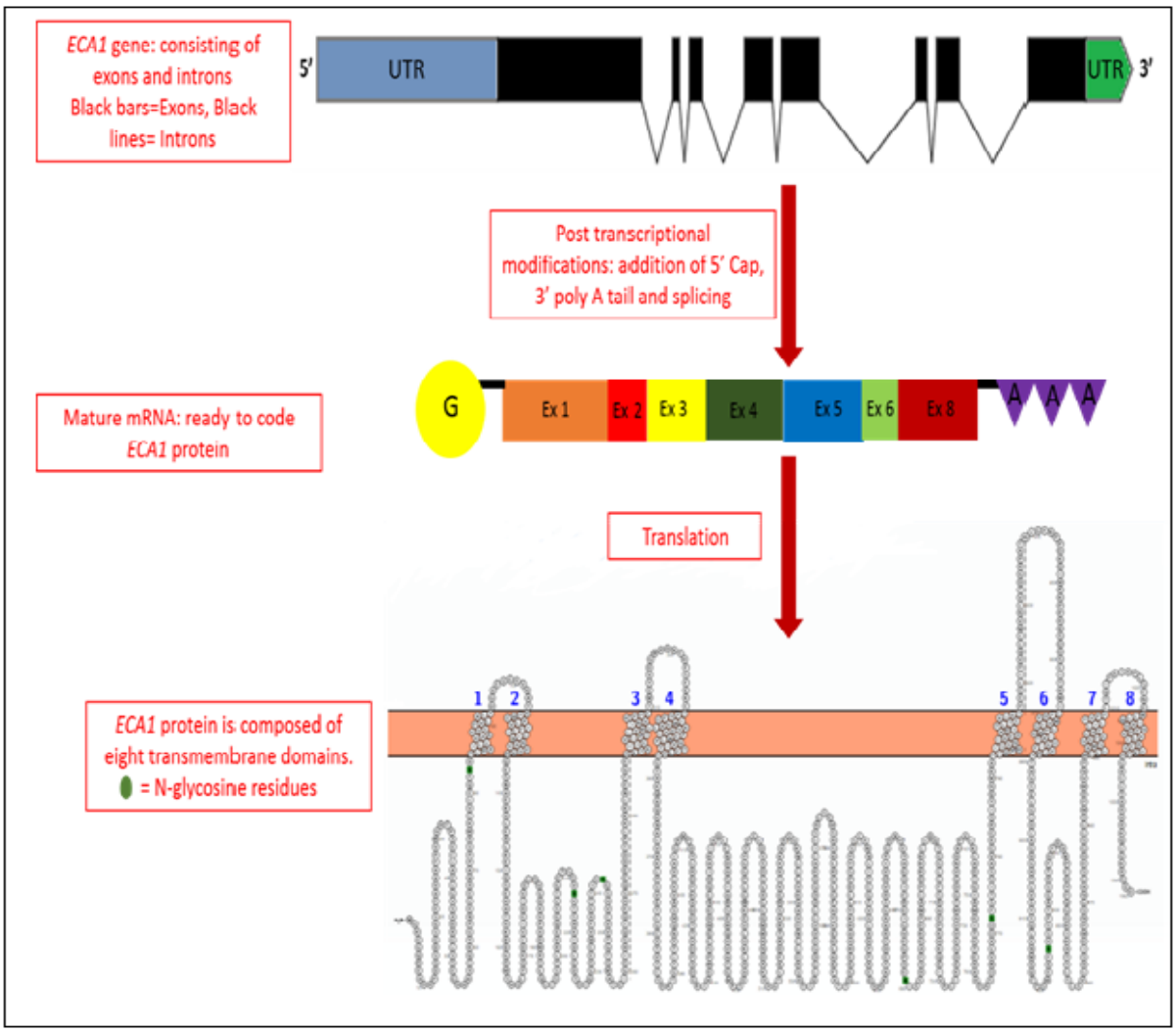

Fig. 1. *ECA1* gene structure in *Arabidopsis thaliana*. *ECA1* gene is composed of eight exons (indicated as light brown), seven introns (indicated as dark brown) and 5' and 3' UTR(indicated as green). The figure is drawn using Exon-Intron Graphic Maker (http://wormweb.org/exonintron) and protter (http://wlab.ethz.ch/protter/start/).

Discussion

P_2 -type ATPases have been found in all the eukaryotic kingdoms ranging from lower animals to higher animals and plants. For example, they are present in Ciliophora and apicomplexan e.g., *Tetrahymena thermophile*, *Plasmodium berghi*. In Mollusca for example, *Pinctada fucata*, in annelids such *Hellobdella robusta*, in Arthropoda such as *Apis mellifera,* etc. Also, they are present in chordates such as *Xenopus tropical*, in fishes such as *Poecilia Formosa*, and higher chordates animals such as *Callithrix jacchus*, *Homo sapiens,* etc.

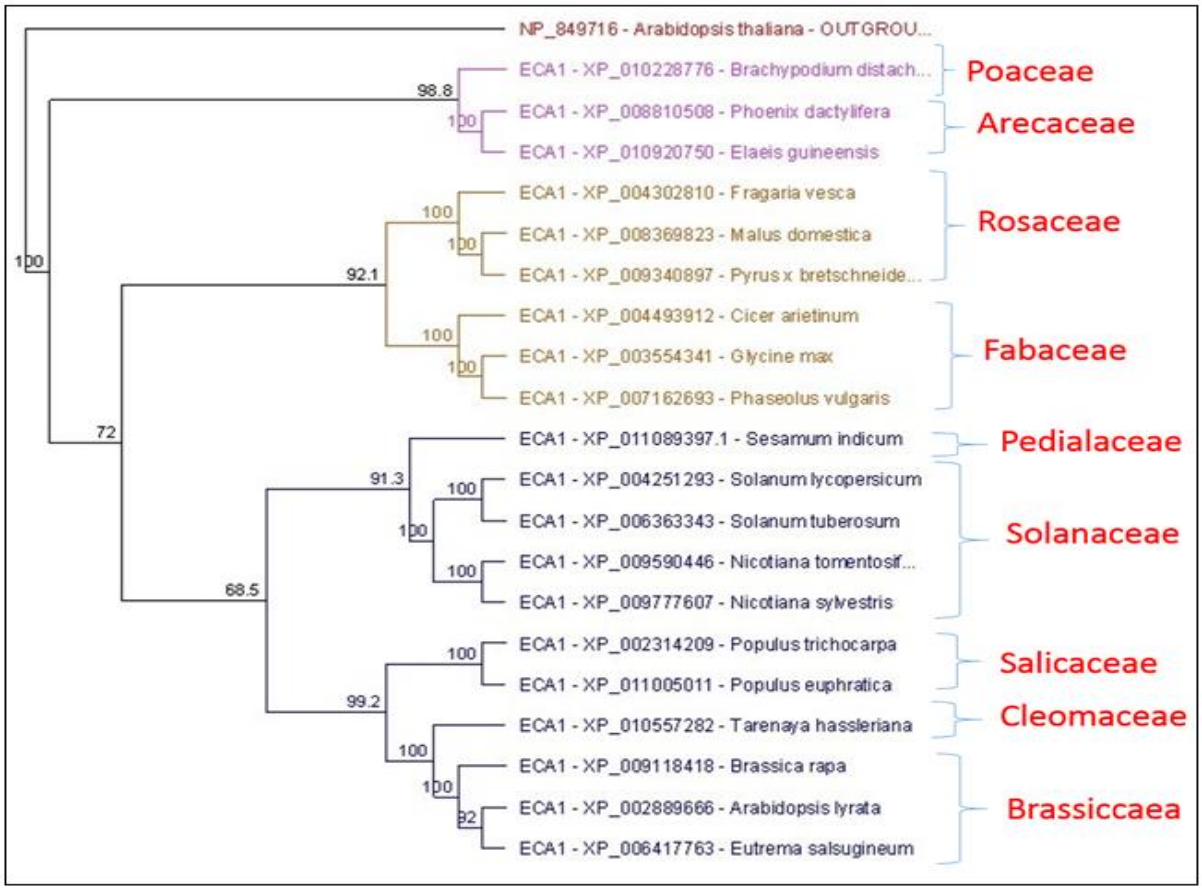

Fig. 2. The evolutionary distances of all isolates of *ECA1* gene from different plant species were computed using the Neighbor-Joining method using HKY Algorithm model. The confidence support value of nodes was estimated by 1,000 replicates of bootstrap. The outgroup sequences used for study in this work has been shown in red. Each clade is shown with a different colour. (A).

They even exist in algae such as *Bathycoccus prasinos* and lower plants such as *Physcomitrella patens*. They are also part of the membrane of higher plants such as dicots (*Arabidopsis thaliana* etc.) and monocots (*Oryza sativa*, *Brachypodium distychon*). Other representative fungal species containing P_2 -type ATPases include *Aspergillus fumigatus, Uncicorpus reesii and Aspergillus niger,* etc (Altshuler *et al.*, 2012).

The presence of this protein in eukaryotes greatly emphasizes the importance of studying the diversity of this protein in different organisms. However, despite its importance, not much work concerning phylogeny has been done on these proteins. Here, we aimed to trace the evolutionary history of *ECA1* (a P_{2A}-type ATPase) in different plant species along with studying diversity in the tertiary structures. In addition to this, we have also predicted tertiary structures of these proteins from different plant families. This analysis helped to understand that evolution of *ECA1* protein is divergent concerning structure while it is convergent concerning function within different plant families. Previous phylogenetic analysis revealed, the separation of *ECA3* protein into various clades according to the kingdom (Altshuler *et al.*, 2012). For example, higher vertebrates such as *Denia rerio*, *Homo sapiens*, *Mus musculus*, *Gallus gallus,* etc. all combine forming one clade (Altshuler *et al.*, 2012). Similarly, tunicate form one separate clade. Animals such as nematode hexapoda, crustacean, nematode, Annelida, Mollusca all combine forming one separate clade. However, concerning plants P_{2A} -type ATPases sequences form two monophyletic clades. *ECA 1*, *2*, and *Arabidopsis ECA4*, were found to be closely related to the clade formed by apicomplexans. Whereas ECA3 gene sequences and is sister to the clade formed by

metazoans. It suggests an earlier gene duplication event in eukaryotic evolution, which led to the loss of *ECA3* like protein from protist and loss of *ECA1*, *ECA2* and *ECA4* proteins from animals and fungi. However, plants retained both copies (Alshter *et al.*, 2012). In this study, the origin of different plant species in relevance to *ECA1* gene was investigated based on a phylogenetic approach. Nucleotide sequence analysis is considered as an accurate method for differentiation and estimation of genetic variations in different plant species. Moreover, in this study, the diversity in tertiary structures of *ECA1* protein in different plant species was also investigated.

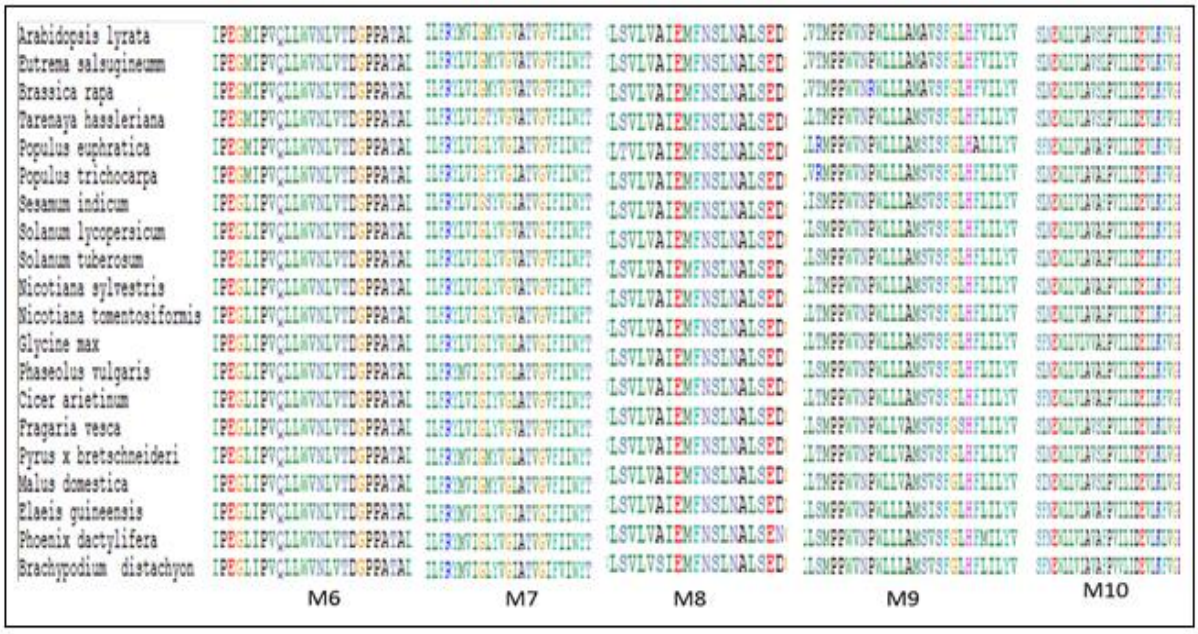

Fig. 3. M1-M10 motifs involved in calcium binding in *ECA1* protein from different species.

Neighbour-joining method for tree construction showed two main clades one consisting of monocots and the other consisting of dicots.

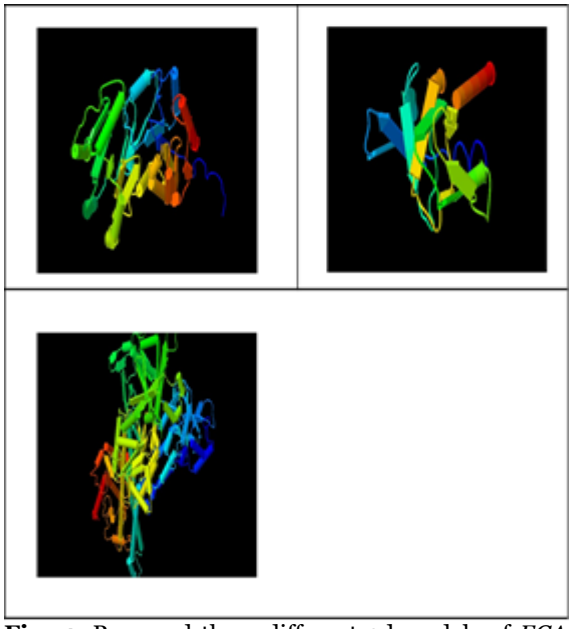

Fig. 4. Proposed three different 3d models of *ECA1* protein in family Poaceae.

This indicates that the evolution of *ECA1* gene has been monophyletic. Clade I (composed on monocots) consist of three species. In this clade *Brachypodium distachyon* branched out separately whereas, *Elaeis guineensis* and *Phoenix dactylifera were* grouped in one clade. This grouping happened due to the close genetic relationships between *Elaeis guineensis* and *Phoenix dactylifera* on basis of the same family i.e., Arecaceae. The clustering of *B. distachyon* along with two species of Arecaceae suggests genetic relatedness between Poaceae and Arecaceae. Similarly, clade II was further divided into two main clades. The first clade was composed of species belonging to the family Rosaceae whereas, the second clade consisted of species from the Fabaceae family. The third clade was subdivided into three clades based on three different families i.e., Salicaceae, Cleomacea and Brassiceae. The last clade (clade IV) was also branched out in two separate clades composed of families Pedialaceae and Solanaceae. The appearance of species in this pattern

suggests the possible relationships between different species based on *ECA1* gene.

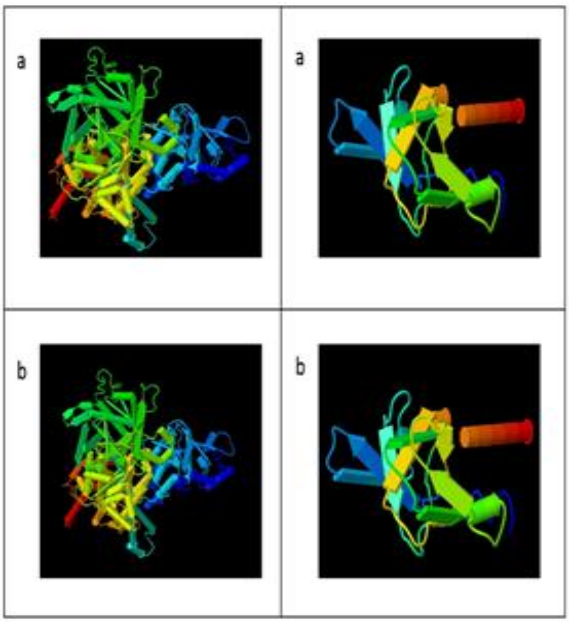

Fig. 5. Proposed three different 3d models of *ECA1* protein in family Arecaceae.

P_2 -type ATPases are known to have conserved sequence motifs that are putatively involved in the binding of metal ions during translocation. Currently, ten such motifs have been identified and they are named as M1-M10 (Pittman *et al.*, 1999). To find out the motif conservation in ECA1 protein from different plant species, sequences (listed in Table 1) were aligned and viewed using BioEdit. All the sequences appeared to consist of all ten important motifs (M1-M10).

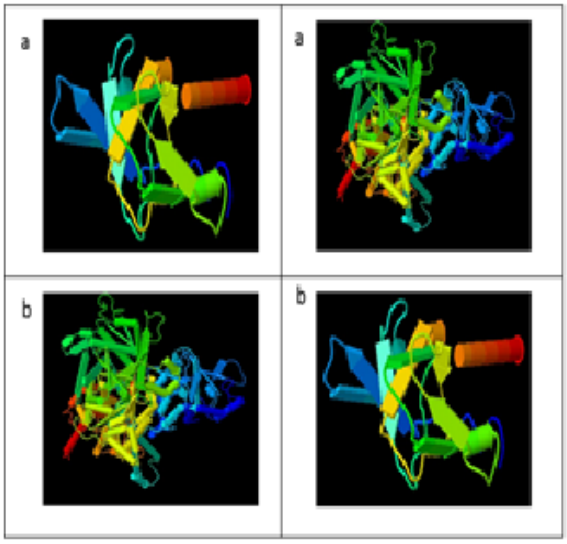

Fig. 6. Proposed three different 3d models of *ECA1* protein in family Salicaceae.

It indicates that evolutionary influences did not cause any change in important sequence motifs that are required for the functioning of these proteins. It also indicated that these plants may evolve from a common ancestor and over time important motifs remained conserved.

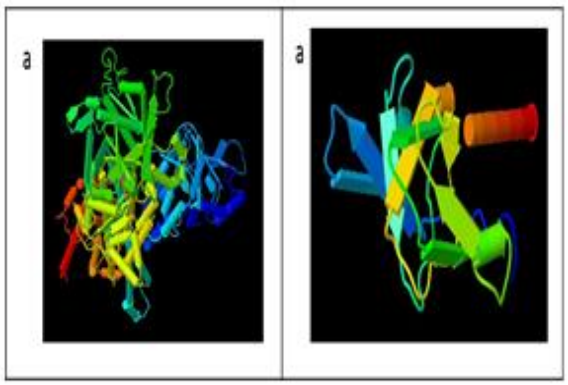

Fig. 7. Proposed three different 3d models of *ECA1* protein in family Pedialaceae.

These results are indicated in Fig. 2. Furthermore, diversity in tertiary structures indicated that mutations had affected sequences, but important sequence motifs had been remained unchanged (Fig. 4- Fig. 7). Therefore, these proteins are structurally diverse but functionally conserved.

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